Thesis for the Master`s degree in Molecular Bioscience Main field of study in Molecular Biology

# Targeting the expression of a putative RNA pyrophosphohydrolase (rppH) gene by artificial microRNAs in *Chlamydomonas* reinhardtii

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60 study points

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# Acknowledgement

The project was performed at the Department of Molecular Biosciences at the University of Oslo under the supervision of Professor Uwe Klein.

I am grateful to Professor Uwe Klein for his guidance and support. I would like to thank Biruk, Anders, and Ragnhild for their support and good times in the lab.

Finally I wish to thank all my friends and family member for their unending support and love.

Pedro Yahyavi
Oslo, May 2013

# Abbreviations

| | |
|---|---|
| Nudix | Nucleoside diphosphate linked to X |
| RppH | RNA pyrophosphohydrolase |
| miRNA | MicroRNA |
| RNAi | RNA interference |
| mRNA | Messenger RNA |
| UTR | Untranslated regions |
| amiRNA | Artificial microRNA |

# Summary

RNA pyrophosphohydrolase (RppH) catalyzes the removal of pyrophosphate from the 5' terminus of bacterial RNAs leaving a 5' monophosphate that is thought to be a prerequisite for RNA degradation. A homolog of the bacterial *rppH* gene has been found in the nuclear genome of the unicellular green alga *Chlamydomonas reinhardtii* by a BLAST homology search. In order to identify the function of the putative *rppH* gene in *Chlamydomonas* artificial microRNAs (amiRNAs) were used to silence expression of the *rppH* gene.

Four amiRNAs corresponding to four different sequences of the putative *Chlamydomonas rppH* transcripts were designed by web-based tools. The four amiRNAs were cloned into a transformation vector in between the promoter and 3' transcription terminator sequences of the *Chlamydomonas psaD* gene and stably inserted into the nuclear genome of the alga.

Selected transformants were screened for the presence of the chimeric *psaD*-amiRNA genes by PCR. Positive transformants were further analyzed by northern analysis for an effect of the amiRNAs on expression of the *rppH* gene. Preliminary results indicate that one of the microRNAs successfully silences expression of the *rppH* gene.

## 1. Introduction



## 2. Materials and Methods



## 3. Result

# 1. Introduction

## 1.1. RNA pyrophosphohydrolase

In *E.* coli RNA pyrophosphohydrolase is thought to initiate RNA degradation by removing pyrophosphate from the 5' end of mRNAs. Removal of pyrophosphate enables RNase E to digest the mRNAs (Figure 1.1).

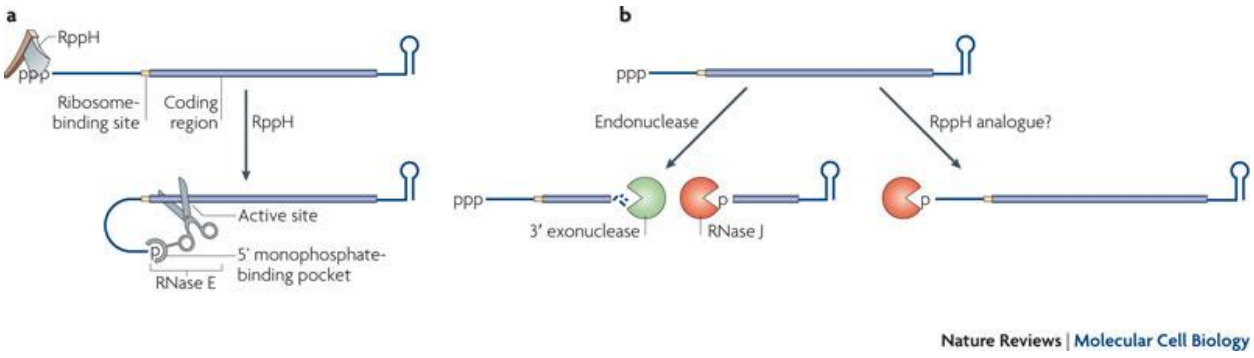


**Figure 1.1: mRNA degradation pathway in bacteria.** a) Bacteria that contain the endoribonuclease RNaseE or a homolog use RppH to remove 2 phosphates from the 5`terminus of mRNAs. This allows RNase E to bind to the 5' end of the mRNAs and start degradation. b) In bacteria containing RNase J an internal cleavage by an endonuclease generates a monophosphorylated intermediate that is susceptible to 5′-to-3′ digestion by RNase J. A pathway analogous to the RppH pathway may also exist in these bacteria. From Belasco (2010).

Plastids in plants and algae harbor a prokaryotic molecular machinery including mechanisms of RNA degradation similar to what is found in bacteria. It is therefore thought that RppH homologs in these organisms are located in plastids where they catalyze the removal of pyrophosphate from plastid RNAs in the course of RNA degradation (Richards, Luciano et al. 2012).
In this project artificial microRNAs suited for silencing RppH expression were introduced into *Chlamydomonas* in an effort to identify the function of a putative *Chlamydomonas rppH* gene.

### 1.1.1. The Nudix motif

RNA pyrophosphohydrolase contains a Nudix motif. Nudix stands for nucleoside diphosphate linked to X. Nudix proteins contain two parts, a Nudix fold beta sheet with alpha helices on either side and Nudix sequence motif or box which contains catalytic and metal binding amino acids (Mildvan, Xia et al. 2005).

Nudix is a motif that uses water to hydrolyze phosphate groups and removes the phosphate from organic compounds. This process is called phosphohydrolysis which is a means to activate or deactivate nucleotides and proteins. Dcp2 of the decapping complex, ADP-ribose diphosphatase, MutT ADPRase, Ap4A, RppH are examples of enzymes that contain a Nudix motif.

Members of this family are recognized by a highly conserved 23-residue sequence, GX (5) EX (7) REUXEEXGU, where U is a bulky hydrophobic residue and X is any residue. The Nudix box contains $Mg^{2+}$ at both the binding and the catalytic site. The enzyme RppH contains a Nudix motif and mediates degradation of mRNA within bacteria (Mildvan, Xia et al. 2005).

RppH is not the only protein containing a Nudix motif within the cell and thus when PCR or Southern blotting is done with Nudix detecting primers or probes, the primer will bind to other Nudix genes as well.

## 1.2. RNA silencing

In RNA silencing or post-transcriptional gene silencing (PTGS) expression of a gene is downgraded or inhibited.
RNA interference is a well-studied method of mRNA silencing. In RNA interference a small 22-24 nucleotide RNA is produced. This small RNA is complementary to a specific region of mRNA and can bind to that region. This prevents ribosomes from attaching themselves to mRNA and preventing the protein synthesis. Tagged mRNA can either be destroyed or remain in an arrested state and to be reversed at a later date. MicroRNA can be separated from the mRNA and mRNA can be activated again in order to produce the protein. When microRNA is removed, ribosomes attach themselves to the mRNA and start the translation (Susi, P., et al. 2004).

RNA interference can occur with microRNA (miRNA) and a small interfering RNA (siRNA). It is believed that the RNA silencing is a method used to combat viral infections (Ding 2000). What distinguishes the miRNA from siRNA is the mode by which it attaches itself to the mRNA and ultimately leads to destruction of mRNA. With siRNAs, a perfect match with a target mRNA marks the duplex for destruction by endonucleases. However; miRNA does not match the target sequence exactly and is unable to distinguish small variations in the recognition sites. As a consequence of that a bulge in the duplex is formed between the miRNA and its mRNA. This leads to two events. First It blocks the target mRNA from translation. Second it protects the target mRNA from destruction by the endonucleases.

Usually miRNA genes are transcribed by polymerase II (Pol II) within the nucleus (Lee, Kim et al. 2004).

Polymerase II produces pri-miRNA. The pri-miRNA has a stem loop, a 5`cap, and a polytail A. This pri-miRNA can contain between 1 to 6 miRNA precursors. Protein Drosha pasha removes the 5`cap and ploy tail A and makes pre-miRNA. The pre-miRNA exported out of the nucleus with Exportin 5. Exportin 5 is a transporter channel that uses GTP bound to RAN protein. Once inside cytoplasm the enzyme Dicer attaches itself to pre-miRNA and unwinds the double helix (Lund and Dahlberg 2006). One of the strands is kept and the other strand is digested (Cenik and Zamore 2011). What determines the selection of the strand is its thermodynamic stability and strong base pairing. The remaining strand stays attached to the Dicer and another protein called Argonaute nuclease. This complex is called RISC (RNA-induced silencing complex) and its role is to identify the targeted mRNA and to bind itself to it and ultimately prevent the translation of the mRNA to a protein by preventing binding of ribosomal RNA (Vermeulen, Behlen et al.2005).

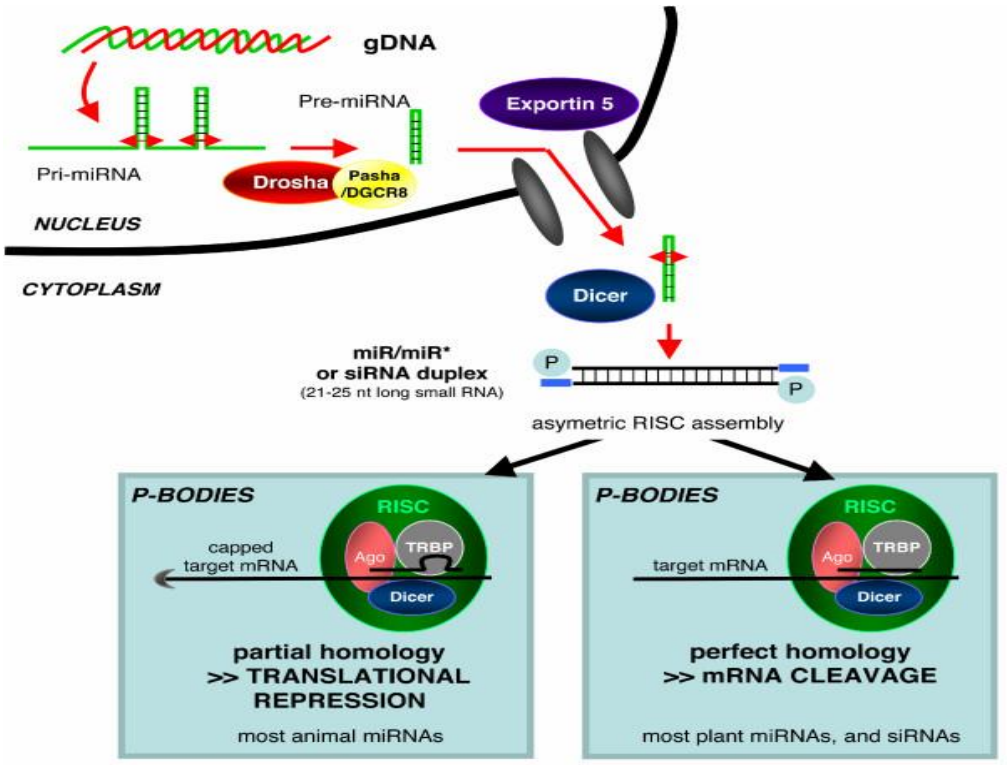


**Figure 1.2: MicroRNA, siRNA structure and function.** MicroRNAs are synthesized by RNA polymerase II and cleaved by Drosha (RNAase III endonuclease) and DGCR8/Pasha in humans (a double-stranded RNA binding protein). This complex recognizes pri-miRNA and cleaves it to release the 60 or 70 bp pre-miRNA. Pre-miRNA is exported into the cytoplasm by exportin-5. Dicer processes the pre-miRNA and RISC complex TRBP (TAR Binding Protein), Dicer and Argonaute (Ago2 in human) guide the microRNA to mRNA. In contrast to siRNAs (small interfering RNA) and plant miRNAs, which induced the cleavage of the targeted mRNA, most animal miRNAs harbor an imperfect homology with their targets and, therefore, inhibit translation by a RISC-dependent mechanism that probably interferes with mRNA cap recognition. This step occurs in cytoplasm foci called P-bodies. From Saumet and Lecellier (2006).

## 1.2.1 mRNA degradation

Life span of mRNA varies in eukaryotic and prokaryotic cells. The life span of mRNA is longer in eukaryotic cells. In eukaryotic cells RNA exosome complex is responsible for processing, quality control and degradation of virtually all classes of RNA (Chlebowski, Lubas et al. 2013).
The RNA exosome complex is made of nine conserved subunits forming the core complex. RNA exosome associates with active ribonucleases, RNA binding proteins, helicases and additional co-factors. In yeast and human exosome complexes are catalytically inactive but in plant they probably remain active (Lange and Gagliardi 2010).

In prokaryotic cells degradosome is responsible for processing rRNA and degradation of mRNA. Degradosome have DEAD box RNA helicaseB, RNAse and polynucleotide phosphorylase. The DEAD box helices unwind parts of the RNA that interferes with degradation. Polyadenylation can promote degradation by creating a toehold for the degradation machinery.

PNPase and RNase E are important in RNA processing and degradation of mRNA in bacteria. Chloroplasts contain homologous gene for both of these two proteins, suggesting that the prokaryotic degradosome is the ancestral origin of the chloroplast (Carpousis et al., 1999). Major portions of mRNAs in the chloroplast are flanked with stem and loop structure 3`s of their coding region that take part in the mature 3`end processing.

Stem and loop structures are necessary for the correct 3`-end processing and mRNA accumulation (Barkan and Stern, 1998).
Chloroplast mRNAs contain poly (A) tails like bacteria and yeast. Poly (A) tails in chloroplast is very long and is about 270 nucleotides; however, poly (A) tails in bacteria and yeast are only 40 to 60 nucleotides long. poly(A) tails in eukaryotic cell are made up of only adenosine versus poly(A) tails in chloroplast are made up of 70% adenosine, 25% guanosines and 5% cytidine and uridine (Lisitsky et al., 1996).
The mRNA that is tagged for degradation is cleaved with endonucleolytic enzymes and produces RNA molecules with no stem and loop structure at the 3`end (B). This fragment is polyadenylated by the addition of a poly (A) tail rich sequence (C).
This polyadenylated mRNA is digested with exoribonuclease PNPase (D) (Lisitsky et al., 1997).

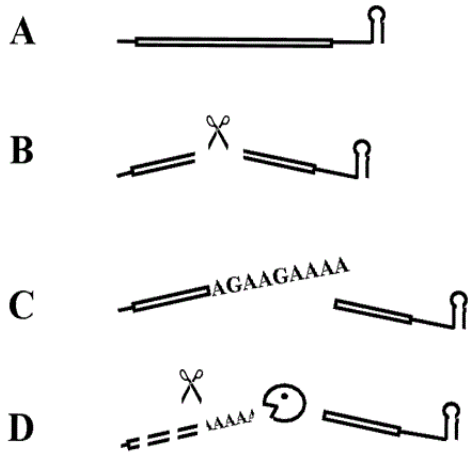


**Figure 1.3: Digestion of mRNA inside the chloroplast.** A) mRNA in chloroplast is tagged for digestion. B) Shows an mRNA which is cleaved with endonucleolytics enzymes. C) Demonstrates the addition of a poly (A) tail rich sequence. D) Depicts a polyadenylated mRNA which is digested with exoribonuclease PNPase, figure adopted from Lisitsky et al., 1997.

## 1.2.2 MicroRNAs in *Chlamydomonas*

What distinguishes the miRNA in animal and plant models is the number of proteins that cleave its precursors. In animal models Drosha pasha cleaves the mRNA inside the nucleus and Dicer cleaves it outside the nucleus. However, in plants Dicer like protein (DL1) performs slicing of mRNA both inside and outside the nucleus. DL1 is only expressed inside the nucleus

of plant cells and is a Dicer homolog. After that the 3`overhang is ethylated by an enzyme called Hua-Enhancer1 (HEN). Another homolog to the exportin 5 called hasty (HST) transports the microRNA into the cytoplasm (Lelandais-Briere, Sorin et al. 2010).

Land plants are thought to be the descendants of green algae-like ancestors (Lewis and McCourt 2004; Palmer et al 2004). The *Chlamydomonas* genome encodes both the dicer and the Agronaute nuclease (AGO) indicating that it is fully competent to perform RNA silencing (Molnar et al. 2007).
Considerable proportions of *Chlamydomonas* miRNA genes are in the introgenic regions; while the remaining mRNA genes reside within the intergenic regions. It is found that multiple *Chlamydomonas* miRNAs can be derived from a single stem and a loop.

The existence of miRNAs in the green alga *Chlamydomonas* raised the possibility that plants and *Chlamydomonas* might be sharing some common miRNAs; however, comparisons between *Chlamydomona*s miRNAs with all known plant and animal miRNAs in the entire data base has found no homologues to miRNAs in Arabidopsis. The lack of universally conserved miRNA genes among all living organisms suggests miRNA genes may have evolved independently in the lineage leading to animal, plant, and green algae (Zhao, Li et al. 2007).

## 1.3 *Chlamydomonas* as a model system

*Chlamydomonas* is a unicellular organism of the green algae family that has two flagella. It is a photosynthetic organism of the family *Chlamydomonadaceae*, genus *Chlamydomonas*. *Chlamydomonas* contains different organelles such as a chloroplast, mitochondria, vacuoles and flagella. The alga is a model organism which is used in studies like motility, chloroplast studies and genetics. *Chlamydomonas* performs photosynthesis through utilizing sunlight and making organic products from inorganic materials. Most of the *Chlamydomonas* species are obligate prototrophs with the exception of C.*reinhardtii* which is a facultative heterotroph. This means *C. reinhardtii* is capable of producing organic substances in the presence of both light and dark. In dark it can use acetate, when present, whereas in the presence of light it can do photosynthesis (Harris 2001).

*C. reinhardtii* is a haploid organism with 17 chromosomes. It has two types of organelles, mitochondria and a single chloroplast. The alga divides by mitosis in favorable times. Under nitrogen deprivation gametes are formed. Two gametes of opposite mating fuse to make a diploid zygote. The zygote remains dormant in the soil until such time when nitrogen becomes plentiful. Then the zygote becomes active and undergoes meiosis and it forms four haploid *Chlamydomonas.*

One of the reasons that *Chlamydomonas* is used as a model organism is its short reproductive cycle and its rapid multiplication. As a model organism *Chlamydomonas* has been modified in many different ways. The strain that was used in this project is the cell wall-less mutant CW-15. Because the mutant lacks a cell wall it can easily be transformed.
Paromomycin sulfate is used as a selection marker. Paromomycin binds to 16S ribosomal RNA and stops translation of proteins (Vicens and Westhof 2001). Only *Chlamydomonas* cells that have incorporated into their genome the vector that harbors the paromomycin-resistance gene are able to grow on paromomycin plates

## 1.3.1. The cell wall of *Chlamydomonas*

The wild type *C .reinhardtii* averages about 10μm in diameter and is enclosed within a cell wall consisting primarily of several layers of hydroxyproline-rich glycoproteins that resemble plant extensions. The cell wall does not contain cellulose to make it hard and inflexible like other plants cells. The CW-15 type of *Chlamydomonas* produces the precursor protein required to form the cell wall in normal quantity but prevents it from assembling into a complete cell wall. Lack of the cell wall makes this mutant susceptible to transformation with foreign DNA and further genetic manipulation (Harris 2001).

## 1.3.2 The chloroplast of *Chlamydomonas*

The chloroplast occupies two thirds of the cell in *Chlamydomonas*. This is where photosynthesis occurs. Photosynthesis leads to production of nutrients; the chloroplast of *Chlamydomonas* contains a 195 kb genome (Harris 2001).

## 1.3.3 Vegetative cell growth

*C. reinhardtii* is capable of both sexual and asexual reproduction. It can grow on agar or liquid medium without co-factors and vitamins. In the absence of light when the strain has access to acetate as a carbon source, energy can be produced.
Wild-type cells grow faster in light with or without acetate in comparison to dark. Optimal growth conditions recommended to grow the algae are a temperature between 20° to 25° in a medium of high salt with adequate lighting (200-400μEinstein's/$m^2$ sec photosynthetically active radiation ). This will support an average doubling time of 6 to 8 hours (Harris 2001).

## 1.3.4 The sexual cycle

Under normal conditions *Chlamydomonas* undergoes mitosis. The cell divides in the dark phase with 2 to 3 rounds of mitosis and makes 4 to 8 daughter cells. Daughter cells are retained inside the cell wall of the mother cell and are released when the cell wall has been digested by a lytic enzyme (Harris 2001).
Under starving conditions such as lack of nitrogen, cells develop into gametes and reproduce sexually. *C .reinhardtii* are haploids (n) and genetically fixed to plus ($mt^+$) and minus ($mt^-$). Under starving conditions the plus and minus gametes make contact with each other, the cell walls are removed and the mating structure is activated. The flagella of the plus and minus cells meet and adhere together and form a complete cell. Subsequently the fused cell loses it flagella and makes a spore. The spore is a diploid zygote (2n) and is ready to germinate under favorable conditions. In the presence of light zygote undergoes the meiosis and releases 4 cells.

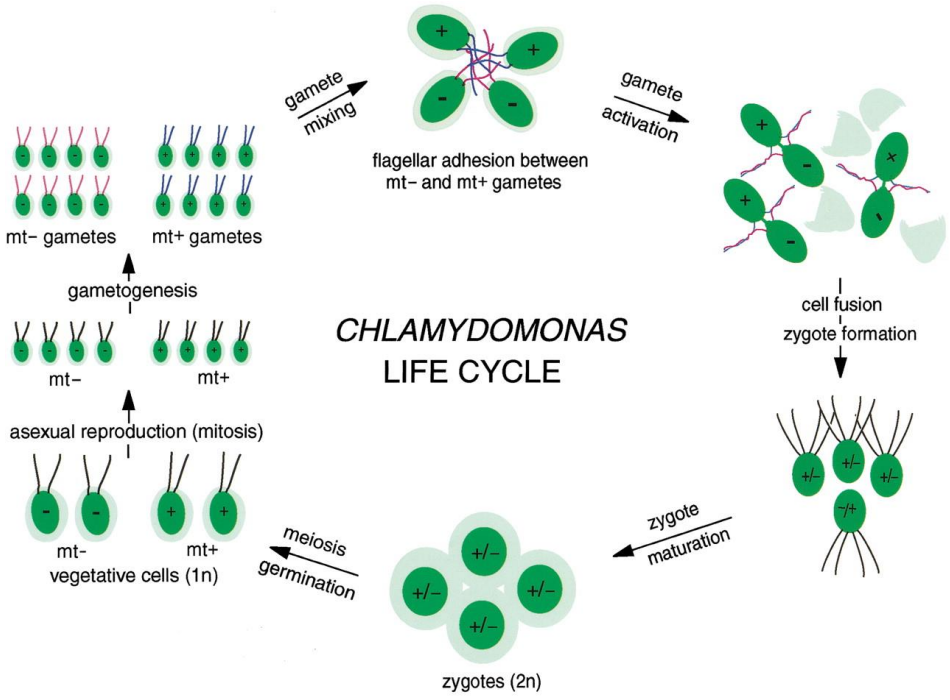


**Figure 1.4: Sexual reproduction of the *Chlamydomonas***. Under starving condition plus and minus gametes make a zygote and reproduce sexually. From Zhao, Lu et al. 2001.

# Aim of the project

The project is based on the hypothesis that a homolog of the bacterial RppH is present in the chloroplast of *Chlamydomonas* where it participates in initiation of RNA degradation. In order to test this assumption we wanted to inhibit expression of the putative *rppH* gene in *Chlamydomonas* using artificial micro RNAs. If successful less RppH should be produced and RNA degradation in the chloroplast should be affected, e.g. levels of mRNAs in the chloroplast could be higher in transformants than in wild-type cells.

# 2. Materials and Methods

## 2.1 MicroRNA

WMD3 (http://wmd3.weigelworld.org) is a site that specifically designs microRNA for different organisms.WMD3 suggests different 21bp DNA strands since DNA is more stable than RNA. Suggested DNA can have different colors; green is the best choice for the microRNA.
A flanking sequence specific to the *Chlamydomonas reinhardii* is added to the 21 bp. Flanking sequence is necessary to make the stem and to give the stem and loop form to the artificial microRNA.
Artificial miRNA resembles the natural RNA. Both start with U, and display 5' instability relative to their miRNA. Their 10th nucleotide is either an A or a U (Schwab, Ossowski et al. 2006).
Certain characteristics associated with siRNA's functionality have been identified and they are: low G/C content, a bias towards low internal stability at the sense strand 3'-terminus, lack of inverted repeats, and sense strand base preferences (positions 3, 10, 13 and 19), (Figure 2.1). Further analysis has revealed that the application of an algorithm incorporating all of the criteria significantly improves potent siRNA selection. This highlights the utility of rational design for selecting potent siRNAs and facilitating functional gene knockdown studies (Reynolds, Leake et al. 2004)

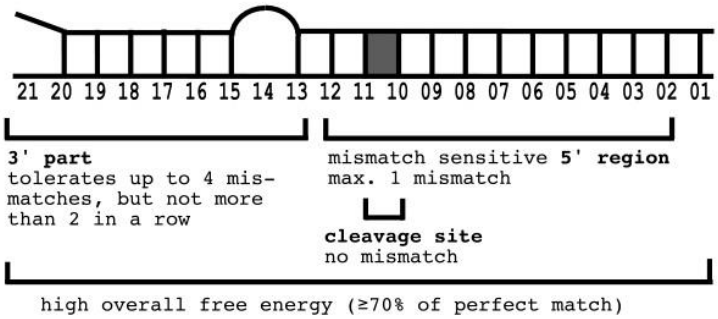


**Figure 2.1: Artificial microRNA mismatches and structure.** In an artificial microRNA the 5`region is sensitive to mismatch but the 3`part can have up to 4 mismatches. Base pairs located at 13, 14 and 15 position must be mismatched too.

To construct the MicroRNA used in this experiment following steps were undertaken:

1- Designer inn (WMD3.com) was accessed
2- The *rppH* gene from the *Chlamydomonas* was inserted in the target gene part.
3- *Chlamydomonas Reinhardtii* from genomic section was chosen.
4- 1 as a Minimum number of included targets was chosen.
5- My E-mail was provided and sends inn was pressed
6- A list containing the best suited MicroRNA was received in a couple of days. The four best suited sequences were selected and were placed in the oligo part of the MWD3 page.
7-for vector choice *Chlamydomonas* was chosen and send inn was pressed.

Shortly after I received a sequence containing MicroRNA and the flanking sequence needed to transform the *Chlamydomonas reinhardtii* with the MicroRNA.
With flanking sequence, oligonucleotides were about 90 base pair and later were sent to the Eurofins Company in order to design the microRNA used in this experiment.
All the suggested microRNA were specific for 3`and 5`UTR

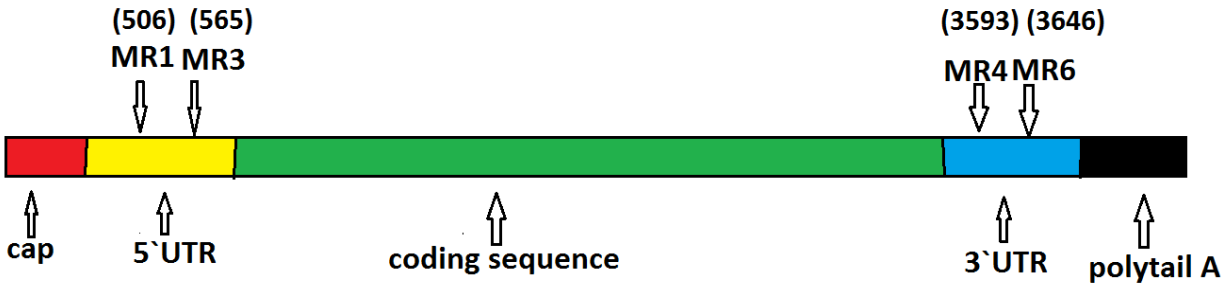


**Figure 2.2: Structure of mRNA contains cap, 5`UTR, coding sequence, 3`UTR and polytail A**. MicroRNA1 and 3 binds to 5`UTR and microRNA4, 6 attaches to 3`UTR. MicroRNA1 attaches to position 506 microRNA3 to 565, microRNA4 to 3593 and microRNA 6 to 3646 in *rppH*

## 2.2 Cloning methods

### 2.2.1 Oligonucleotide cloning

-Mix 5 µl of each of the single stranded oligonucleotides (100pmol/µl) with 30µl of sterile water (total volume 40µl).
-heat for 2 minutes at 100°C.
-collect liquid on the bottom of the tube by centrifuge (room temperature) and let it cool down slowly (15 min) on bench.
-add 5µl of poly nucleotide kinas buffer (10X) and 5µl ATP (10mM, pH 7) and mix
- add 1 µl of T4 polynucleotide kinas, mix again and spin briefly and incubate at 37ºC for 60 min
-Run the Oligonucleotides on the 1.3% agrose gel.
-Weigh a DNAase free 1.5 ml eppendorf
-use a scalpel and a large wavelength to cut the band from the gel
-weight the eppendorf tube with gel.
-add 10µl of capture buffer type 3 for each 10 mg of gel.
-mix and incubate at 60°C for 15 min to dissolve, shake the mixture every 3 min.
-once the gel is dissolved check the color of the capture buffer 3 to ensure it is still yellow.
-place one GFX micro spin column into one of the collection tubes
-Transfer DNA mix in GFX column within the collection tube.
-Incubate at RT for 1min.
-Spin the assembled column and collect tube at 1600 rpm for 30 sec
-Discard the flow through.
-add 500 µl of wash buffer type 1 to column.
-spin the column for 30 min in 1600g.

-Transfer GFX to an eppendorf tube.
-Add 20µl of elution buffer type 6 to GFX column
-wait 1 min in RT and spin in 1 min in 1600g.

## 2.2.2 Dephosphorylation

-Linearized Sk+ vector is ethanol precipitated.
-Add 90µl of water to dry pellet.
-Add 10µl buffer 10X dephosphorylation buffer
-Add 1µl CIP (calf intestine phosphate), mix and incubate at 37°C. Add the same amount 100µl Phenol/chloroform /isoamyl alcohol [25:24:1], vortex and centrifuge transfer the upper phase to a new tube.
-Add the same amount of Chloroform /isoamyl alcohol [24:1], vortex and centrifuge transfer the upper phase to a new tube.
-Precipitate DNA by adding 1/10 of the volume Na-acetate (3M) and 2 volumes ethanol. Leaves tube at freezer or dry ice for 30 min.
-Collect the Precipitate by centrifuge (10 min, 4 min), wash the pellet with ice cold 70 % ethanol and dry.
-Dissolve DNA at 20µl water.
-Add the same amount of 100µl Phenol/chloroform /isoamyl alcohol [25:24:1],
vortex and centrifuge then transfer the upper phase to a new tube.
-Add the same amount of Chloroform /isoamyl alcohol [24:1], vortex and centrifuge and transfer the upper phase to a new tube.

-Precipitate DNA by adding 1/10 of the volume of Na-acetate (3M) and 2 volumes of ethanol. Leave tube at freezer or dry ice for 30 min.
-Collect the Precipitate by centrifuge (10 min, 4 min), wash the pellet with ice cold 70 % ethanol and dry.
-Dissolve DNA at 20µl of water.

## 2.2.3 Ligation mix

-Add the insert to eppendorf tube
-Add appropriate amount of the vector
-Add water to final concentration of the 6.5µl
-Incubated for 45°C for 5 min put on ice.
-Add 1µl of ligase buffer, 2µl of PEG (remove water), 0.5µl of ligase T4 to the mixture
-Incubated at 19°C for 3 hours.

### 2.2.8 Transformation of *E.coli*

-Add 3µl of ligation mixes to the competent cell and keep on ice for 30 min . -Heat shock the cell for 1 min at 42 °C

-Add 0.8ml LB to mixture and keep at 37 °C for 1 hour. Plate 75 µl of the mixture on LB + ampicillin plate and keep over night at 37 °C.

## 2.2.4 Transformation of *E.coli*

-Add 3µl of ligation mixes to the competent cell and keep on ice for 30 min . . Heat shock the cell for 1 min at 42 °C

-Add 0.8ml LB to mixture and keep at 37 °C for 1 hour. Plate 75 µl of the mixture on LB + ampicillin plate and keep over night at 37 °C.

## 2.2.5 Small Scale isolation of plasmids from *E.coli* TB1 cells (Mini prep)

-Pipette 1.5 ml of each culture into the microfuge tube. Spin for 20 sec at full speed
-Remove the medium with a pipette leaving the bacterial pellet as dry as possible.
-Add 100µl of ice -cold TEG buffer and resuspend the pellet by vigorous mixing.
-Leave the tubes 5 min at RT. During this time , prepare a solution of 0.2 N NaOH /1% SDS by gently mixing in a eppendorf tube 800µl of destilled water with 100µl of NaOH[2N] and 100µl of SDS [10%]. Keep at RT.
-Add 200µl of the 0.2N NaOH /1%SDS solution to each tube. Mix by inversion. Do not vortex. Incubate 5 min on the ice.
-Add 150µl of ice cold potassium acetate [5M, pH 4.8]. Mix by inversion for about10 sec. Do not vortex. Incubate on the ice for 5 min.
-Centrifuge in a cold (4°C) microfuge 5 min at max speed.
-With pipette transfer the supernatant to a new tube avoiding the white precipitate on the side of the tubes.
-Add 410µl of phenol/chloroform /isoamyl alcohol [25:24:1] into the supernatant .Mix by vortaxing (mixture will turn milky white) and centrifuge for 2 min at RT at max speed to separate the phases.
-Transfer the upper phase (containing the plasmids) to the new tube and add 410µl of Chloroform /isoamyl alcohol [24:1]. Mix by overtaxing and centrifuge for 2 min as before.
-Transfer 310µl of the upper phase to a new tube. Add 750µl of ice cold 96% ethanol. Mix and precipitate nucleic acids for 10 min on ice.
-Centrifuge 10 min in the cold at max speed.
-Remove and discard the liquid with a pipette and add 1 ml of ice-cold 70% ethanol to the pellet. Mix by inversion and centrifuge in the cold for 5 min as before.
-Remove the supernatant leaving the pellet as dry as possible. Leave the pellet at RT to let t air dry or vacuum dry.
-Dissolve the dry pellet with pipette in 15µl of sterile distilled water.

## 2.2.6 Maxi prep

Maxi prep or *E.coli* plasmid preparation by CsCl gradient centrifugation
-Grow 100ml of LB with 100µl ampicillin (1µg/µl) culture over night at 37°C shaking.
-Collect the cells by centrifugation for 5 min at 4°C at 5000 rpm.
-Resuspends cells in 3.6 ml of cold TEG (25mM Tris (pH 8), 10mM EDTA, and 50mM glucose) and remove cells to 50 ml tubes.
-Add 0.4 ml of 10 mg/ml lysozyme (0.01 g lysozyme/1ml TEG) in TEG (prepared fresh) to the cells and leave at RT for 5 min and move to an ice bath for an additional 5 min.
- Add 8 ml of 0.2 N NaOH/1% SDS (prepared fresh), invert to mix and leave on ice for 5 min.

-Add 6 ml of cold 5 M potassium acetate (pH 4.8), invert to mix, and leave on ice bath for 5 min.
-Centrifuge in SS-34 rotor at 6K for 10min at 4°C to pellet debris.
-Pour supernatant through cheesecloth into a 50 ml tube and add 12.5ml of isopropanol.
-Leave at RT for 15 min and centrifuge in SS-34 rotor at 10K for 10 min at RT.
-Aspirate supernatant thoroughly and add 3 ml of 50 Tris (pH 8)/1mM EDTA to the pellet.
-Resuspends the pellet and TE to 4.2 grams in 30 ml tube.
-Add 4.5g CsCl and mix until the pellet is dissolved and allow solution to warm to RT.
-Add 0.5 ml of 10 mg/ml ethidium bromide, mix and centrifuge in SS-34 rotor at 4K for 5 min at RT.
-Remove supernatant to a Vti80 tube and heat seal.
-Load rotor and centrifuge at >50K for more than 15 hours at 15°C.
-Decelerate the mixture without brake, extract band with 3 ml syringe and 16 gauge needles.
-Remove EtBr by repeated (4-5X) extraction with isopropanol /water (7:1).
-Dialyze against 1 liter of sterile TE at 4°C for >3 hours, replace buffer with fresh 1 liter sterile TE and leave overnight.
-Find the concentration with nano drop.

## 2.2.7 Cloning of microRNA

Oligonucleotides were ordered from Eurofins MWG operon. Water was added to the oligonucleotides to make the concentration of the DNA 100 pmol/µl. Oligonucleotides are single strands and have to be double stranded for it to be cloned within the vector. Oligonucleotide cloning method was used to purify the DNA and to make it complementary.

Linearized SK+ vector was precipitated with ethanol precipitation method and dephosphorylated. To ligate the microRNA inside the vector, appropriate amount of the vector and insert were added together. Mixture incubated at 45°C for 5 minutes and then placed on ice.
2µl of PEG (removes water), 1µl of ligase buffer and 0.5µl of ligase T4 were added to the mixture of the insert and SK+ vector. Ligase mix was incubated at 19°C for 3 hours.
Competent *E.coli* was transformed with 3µl of ligation mix, plated and kept overnight.
Four colonies were picked up from the plate, and mini preps were performed on them to isolate the plasmids.
Plasmids from mini prep were checked to make sure they contain the insert and specifically to ensure that insert sites were in the right direction (5` to 3` direction).

3µl of plasmids were cut with AfeI restriction enzyme. Plasmids without the insert were not cut and remained supercoiled in the gel. The plasmids containing the insert were cut and linearized. MicroRNA was inserted in 5` to 3` or 3` to 5 ` direction. Plasmids with insert (linearized with AfeI digestion) were further cut with SpeI and XbaI. The plasmids containing the insert in the right direction is about 160bp versus the one in the wrong direction is about 230 bp.
Plasmids containing the microRNA were used to inoculate 100 ml of LB with ampicillin. Maxi prep was used to isolate plasmids in a much larger quantity.
Appropriate amount of the SK+ (10000ng) was digested with XbaI and XhoI, Insert was placed between XbaI and XhoI site. SK+ digestion resulted in a fragment which is about 1200 bp. This fragment was then ligated within the pChlamiRNA3int vector (plasmids). Plasmid pChlamiRNA3int is 6481bp which contains Paromomycin sulfate resistance gene is used to

select the with the insert.
Plasmid pChlamiRNA3int was digested with the XbaI and XhoI. Digested plasmids ran on the gel. Electrophoresis of the digested material formed two bands. The smaller band was discarded while the larger band was isolated from the gel.
Using transformation method SK+ plasmids (~1200bp) was ligated within the pChlamiRNA3int (~4600bp).The transformed Product was the pChlamiRNA3int with micoRNA. Plasmids were cloned within the *E.coli* genome and then ran through the mini prep. Plasmids were cut sequentially with the AfeI, and then with XbaI and SpeI enzymes to ensure that plasmids contain the insert and that the insert is inserted in the right direction. Sample with the right insert was selected and inoculated in 100ml of LB+amp. Plasmids were isolated with the maxi prep and dialyzed and later ethanol precipitated for purification. The Purified plasmids were dissolved in 20µl of water. To find the concentration of plasmids nano drop was used. Plasmids (10000ng) were digested with KpnI enzyme in order to make them linearized for transformation of *Chlamydomonas*.

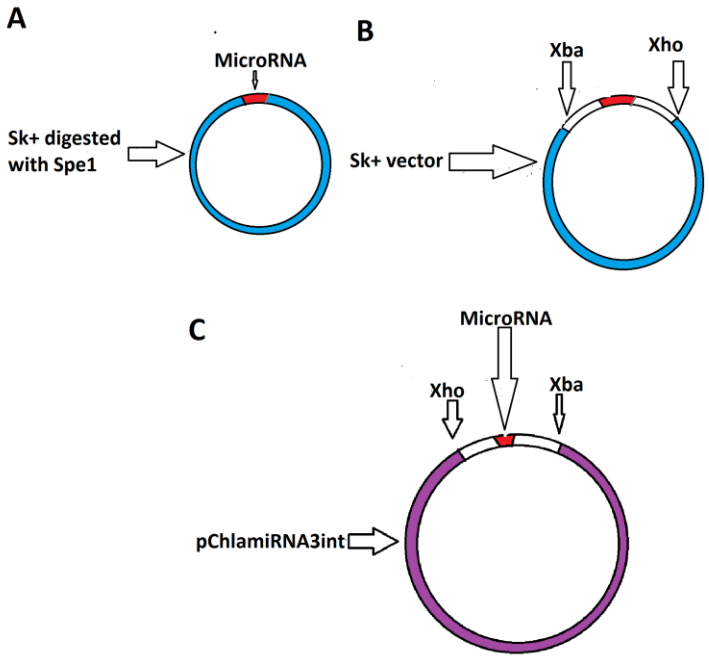


**Figure 2.3: Cloning step for microRNA.** A) MicroRNA is inserted within SK+ vector after it has been digested with SpeI. B) SK+ vector is further digested with XhoI and XbaI and fragments containing microRNA is separated. C) Fragments are inserted within the pChlamiRNA3int vector.

## 2.3. Nuclear Transformation

MicroRNA is inserted inside the pChlamiRNA3int and transformed into the *C .reinhardtii* cell. Since *Chlamydomonas* cell does not have a cell wall it can be transformed easily. Plasmids are cut with KpnI in order to make them linearized and to transform the cell. Plasmids are inserted within the nuclear genome.

The shotgun method can be used to do this, but our experiment method is cheaper, easier and more effective. If plasmids are inserted inside the genome, the transformation is permanent and the cell will never loses the insert.
Linearized pChlamiRNA3int with microRNA was transformed within *Chlamydomonas* with no cell wall (CW-). 40 ml of *Chlamydomonas* was centrifuged and the supernatant was removed. The remaining pellet was dissolved in 0.5 ml of HS solution. To make the transformation mix, 337µl of *Chlamydomonas*, 300µg of beads, 66µl of TEG and 7.5µl of DNA were added and vortexed for 15 seconds. The solution was removed and plated on the plate with paromomycin. Then the plate was left undisturbed for over 2 weeks.
If the *Chlamydomonas* is not transformed with the vector it cannot survive. However; if it is transformed with the pChlamiRNA3int vector it can grow on the plate. The pChlamiRNA3int with the insert was transformed within the *Chlamydomonas* and was integrated inside the genomic DNA of *Chlamydomonas* and began to produce the paromomycin and microRNA. Colonies from the plate were later transferred to a medium of HS and placed under the light.

After 30 days, colonies started to grow in the HS solution. To speed the growth of *Chlamydomonas* the solution was transferred to the $CO_2$ chamber. DNA isolation methods were used to isolate the DNA from *Chlamydomonas*.

## 2.4 Work with *Chlamydomonas*

### 2.4.1 Isolation of genomic DNA from *Chlamydomonas*

-Spin down (5 min 4000 rpm, SS-34 rotor) 40-50 ml of *Chlamydomonas* culture.
-Resuspend the pellet in 0.75ml of DNA extraction buffer (100mM Tris pH 8.0; 50mM $Na_2$-EDTA; 0.5M NaCl; 10mM β-mercapto ethanol) and transfer suspension to a 2ml microfuge tube.
-Add 60µl of SDS (21%), mix, and incubate the mixture for 15 min at 65°C.
-Let the mixture cool down on RT and add 0.7 ml of phenol (equilibrated with 0.1 M Tris, pH 8.0). Mix carefully by inverting the tube and spin for 5 min in microfuge at RT.
-Transfer supernatant to a new microfuge and add 0.750ml Phenol/chloroform /isoamyl alcohol [25:24:1], and mix and spin.
-Transfer 0.650 ml of supernatant to a new 1.5 microfuge tube and add equal volume of isopropanol. Mix by inverting the tube and letting it stand at RT for 5 min.
-Collect the precipitated nucleic acids at the bottom of the tube at low speed centrifugation (2000rpm) at RT if DNA is visible you can remove it with glass or pipette tips.
-Discard as much supernatant as possible and wash pellet in a 1ml ice cold 70% ethanol. To dry the pellet spin for 2 min in full speed.
-Resuspend pellet in 90µl of Te buffer (10mM Tris, pH 8.0; mM EDTA), add 10µl of RNase a (1mg/ml), and incubate at 37°C for 1 hour.
-Extract the mixture once with 100µl Phenol/chloroform /isoamyl alcohol 25:24:1] and once with 100µl Chloroform /isoamyl alcohol [24:1].
-Precipitate DNA in freezer with 0.3 M Na-acetate /2 volumes ethanol.
-Collect DNA precipitate in microfuge in cold, wash with ice-cold 70% ethanol and dry the pellet.
-Resuspend the DNA in 20µl of sterile water. DNA concentration will be ~200 ng/µl, and yield of ~4µg.

## 2.4.2 RNA isolation from *Chlamydomonas reinhardtii*

-Centrifuge at 5000rpm at 4°C for 5 minutes 40 ml of *Chlamydomonas* culture containing 2X106 cells /ml.
-Re suspends the pellet in 1.5 ml of ice-cold lyses buffer (0.6mNaCl, 200mMTris pH8.0, and 10mMNa2-EDTA); add 150µg of RNAase inhibitor (200mM vandal rib nucleoside (NEB)).
-Pipet cells into a 15ml sarstedt tube containing 2 ml of phenol and 1.5 ml of SDS (4%) preheated to 65°C. Cap and mix.
-Incubate at 65°C for 15 min (mix occasionally [3-4 times] by shaking).
-Cool down on ice and add 1ml of ice cold Chloroform /isoamyl alcohol (24:1) and Mix.
-Centrifuge at 7000rpm for 5 min (SS34, 4 °C).
-Take 3 ml of the supernatant and transfer to another 15ml sarstedt tube containing 3ml of ice cold Phenol/chloroform /isoamyl alcohol [ 25:24:1]. Mix and spin as before.
-Pipet 2.5ml of the supernatant into the tube containing 2.5ml of ice cold isopropranolol and 250µl of 3M Na-acetat (pH5.2) .Mix and incubate at -20°C for at last 1 hours ( over night is better).
-Spin for 15 min at 12000rpm. Decant supernatant; watch pellet it as it may get loose. Pellet should be almost dry; Re suspends the pellet in 300 µl of DEPC -treated water and transfer to a sterile 1.5ml Eppendorf tube containing 100µl of 1ice- cold 8m LiCl. Mix and incubate on ice for 2 hours.
Spin in microfuge at 4°C for 30 min. Remove supernatant with a pipet and resuspend the pellet in 100µl of DEPC treated water. Keep on ice.
Dilute 10µl of RNA solution in 1 ml of DEPC treated water and measure $OD_{260nm}$ (OD1.0 =40µg RNA/ml). To the rest of the RNA solution add 10µl of 3M Sodium acetate (pH5.2) and 200µl of ice cold ethanol. Mix and incubate at -20°C for 1 hour. Spin in microfuge in a cold room for 10min.
-Wash pellet with 1 ml of ice cold 70%ethanol (10 min spin). Store the RNA in 70 % ethanol if it is not used immediately .Dry pellet briefly in a vacuum centrifuge. Resuspend RNA at a concentration of 2µg/µl in DEPC -treated water.

## 2.5 Analytic methods

## 2.5.1 PCR

Genomic DNA was used as the template. In the experiment 100ng of genomic DNA was added to 50µl of PCR solution. Primer 5`paro4331 and 3`paro4743 was used at the annealing temperature of 59°C .The Product was about 412 bp which proves paromomycin is present within the genome as the Primers used were specific for paromomycin.
A new PCR on the samples containing paromomycin gene was performed using new primer 3`psaD495 and 5`psaD6401 at annealing temperature of 63°C. The Primers used at this step were specific for promoter region in the vector and the product was around 713bp.

Primer5`interon and 3`psaD495 at annealing temperature of 62°C was used on the sample containing paromomycin and promoter region. Primers are specific to intron in the vector and promoter region in the vector and can give different bonds. Our targeted Product must be around 543bp.

## 2.5.2 Southern blotting

Southern blotting utilizes DNA interaction between probe and DNA to detect a specific sequence within the genomic DNA. In this experiment a probe for detecting the paromomycin sequence was used to detect the paromomycin gene in the genomic DNA. PCR with paromomycin primer proved paromomycin was already present within the genomic DNA and southern blotting used to confirm the PCR result. Southern blotting is another common method used in laboratories. It was performed to reconfirm the PCR result.

Plasmid pChlamiRNA3int was digested by BamHI and XhoI enzymes. After digestion was completed, the product was fractionated on the gel and an 1800bp fragment containing paromomycin gene was isolated from the gel. The probe was prepared by Professor Uwe Klein using the 1800bp fragment.
Isolated genomic DNA was digested by Bathe and XhoI and ethanol precipitate.

-Resuspend the DNA in 20µl of water, including DNA loading gel buffer.
- Run the reaction on the agarose gel.
-Check the gel under UV light and take a photo.
-Transfer DNA to nylon membrane according to protocol
-Put the membrane in SSC [2X] .Check gel under UV – light for complete transfer.
-Wrap the membrane into plastic Wrap and cross link DNA to membrane with CL-1000 ultraviolet cross linker, UVP (USA), set to 1500 energy.
-Hybridize the membrane with a radioactive probe and develop by auto radiography.

## 2.5.3 Northern blotting

Northern blotting is utilized to detect RNA using complementary DNA strands. *Chlamydomonas* RNA was isolated using RNA isolation method and was subsequently added to paromomycin probe to detect the mRNA produced by the *Chlamydomonas* containing paromomycin resistance gene. Probes specific for Nudix was used to detect Nudix mRNA within the pool of mRNA.
-Wash the tray and comb in the 3% $H_2O_2$ overnight.
-Dissolve 0.78 g agarose in 37ml DEPC treated water (1.3% agarose gel). Cool down in water bath at 65°C.
-Add 12ml of MOPS buffer [5X], and 11ml of formaldehyde [37%].
-Mix gently by swirling and pouring into a gel trey: Put in the comb.
-Prepare the samples by mixing every sample in a microfuge tube:
2.5µl of DEPC treated water
2.0µl of MOPS buffer [5x]
3.5µl of formaldehyde

3.5µl of ethidium bromide [100µg/ml]
10µl of formamide
2µl of RNA sample [2µg/µl]
-Incubate samples at 65°C for 15 min.
-Put the gel into the electrophoresis chamber and add running buffer to cover the gel. (315ml of

DEPC treated water, MOPS [5X] and 45 ml of formaldehyde).
- Pre run for 5 minutes at 60mA.
-Cool down samples. Spin in centrifuge at maximum speed for 1 min and add 2µl of RNA gel loading buffer.
-Mix with pipette and add 20µl of sample for every well.
-Run the sample at 60mA for 10 min in reverse direction, then in normal direction until the bromphenol blue bands is at the bottom of the gel.
-Take a photograph from the gel.
-Wash briefly in DEPC treated water and transfer RNA to a nylon membrane following the protocol.
-Transfer for 5.5 hours. Put membrane into SSC [2X].Check gel under UV light for complete transfer.
-Wrap the membrane into plastic wrap, and cross link DNA to the membrane with CL-1000 Ultraviolet Cross linker, UVP, set to 1500 energy.
-Hybridize the membrane with a radioactive probe and develop by auto-radiography.

# 3. Results

Four different artificial microRNAs were used to silence the *rppH* gene (see Appendix 5). These microRNAs were supposed to bind to transcripts of the putative *rppH* gene in *Chlamydomonas* in the 5' UTR region (microRNAs 1 and 4) and in the 3' UTR region (microRNAs 3 and 6). The four microRNAs were chosen from a list of possible microRNAs that was provided by the WMD3 - Web MicroRNA Designer tool (http://wmd3.weigelworld.org) using the putative *rppH* gene from *Chlamydomonas* as the target sequence. Besides suggesting sequences for microRNAs the tool provides also the sequence of an oligonucleotide (≈ 90 nt including the microRNA sequences) that can be directly cloned into the *Spe*I site of the pChlamiRNA3int transformation vector (Molnar, Bassett et al. 2009) .

## 3.1 Cloning of the microRNAs into the transformation vector

Complementary ≈ 90 nt oligonucleotides, obtained from Eurofins MWG (Ebersberg, Germany), were annealed (Materials and Methods) and purified on an agarose gel (Figure 3.1).

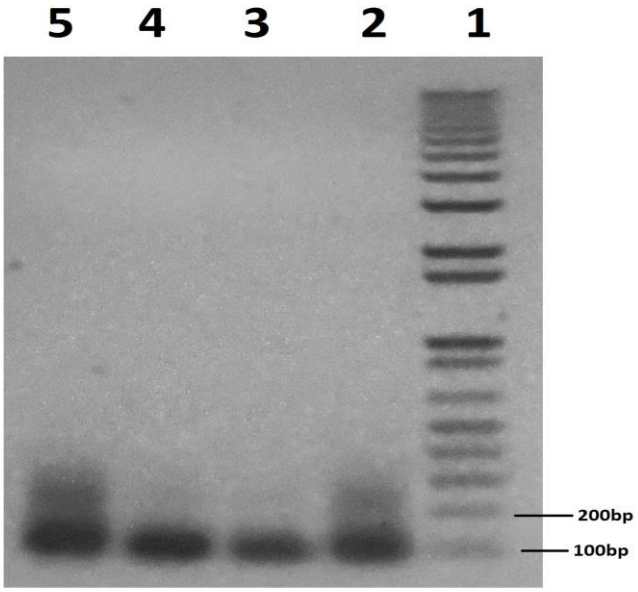


**Figure 3.1**: **Purification of annealed oligonucleotides.** Lane 2, 3, 4, 5 show the four microRNAs purified on a 1% gel. The bands were cut from the gel and purified with GFX PCR DNA gel band purification kit (GE Healthcare). Lane 1 contains the DNA ladder, lane 2 contains microRNA1, lane 3 contains microRNA3, lane 4 microRNA4, and lane 5 contain microRNA6.

The purified oligonucleotides were cloned into the *Spe*I site of plasmid psaD/SK+, a pBluescript SK+ vector into which the 1200 bp *Xho*I-*Xba*I fragment from the pChlamiRNA3int vector (Appendix 2) has been cloned. The *Xho*I-*Xba*I fragment contains the *Chlamydomonas psaD*

promoter and 3' terminator sequences that flank the *Spe*I site into which the oligonucleotides were inserted. The total size of the psaD/SK+ vector is about 4200 bp.

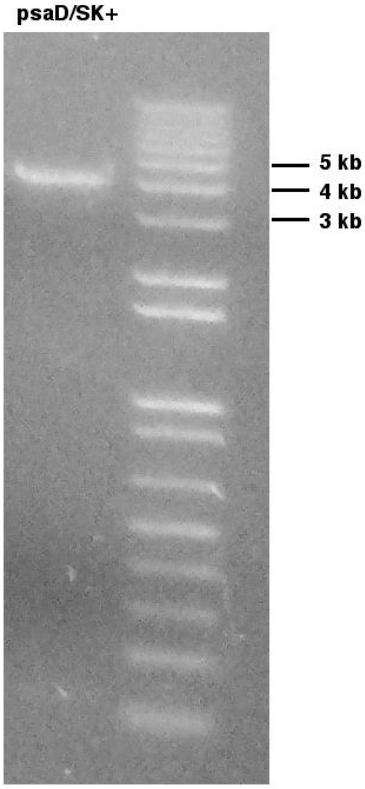


**Figure 3.2: psaD/Sk+ vector** digested with SpeI (lane 2).

Prior to cloning the linearized psaD/SK+ vector was dephosphorylated to avoid religation. Competent *E.coli* TB1 cells were transformed and minipreps of colonies screened for the presence of the oligonucleotides by digestion with *Spe*I and *Xba*I (Figure 3.3). Positive transformants released a fragment of ≈150 bp.

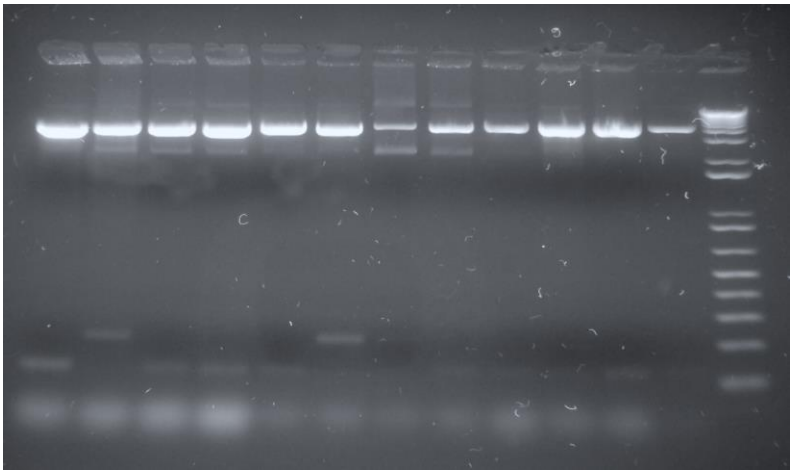

**Figure 3.3: Example for screening of transformant DNA for the presence and orientation of microRNA-containing oligonucleotides.**
Miniprep DNA was digested with *Spe*I and *Xba*I releasing a fragment of 150 bp or 250 bp depending on the orientation of the oligonucleotide in the vector.

In addition to digesting the miniprep DNA with *Spe*I and *Xba*I positive samples were also digested with *Afe*I (not shown) because the oligonucleotides contained a unique *Afe*I site. Plasmids that did not contain microRNA were not digested by *Afe*I and remained supercoiled.

Plasmids were amplified and isolated by maxi preps, digested with *Xho*I and *Xba*I (Figure 3.4) and cloned into the *Xho*I/*Xba*I sites of the final transformation vector pChlamiRNA3int (Appendix 2).

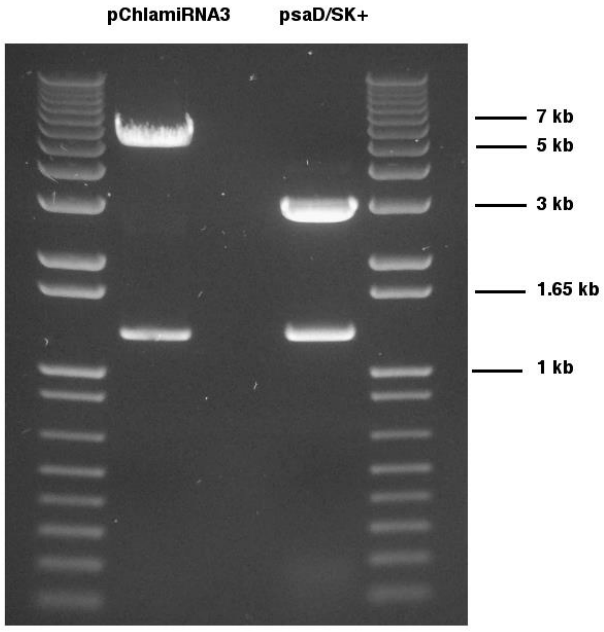


**Figure 3.4: Plasmid psaD/SK+ and the final transformation vector pChlamiRNA3int digested with *Xba*I and *Xho*I.**
The smaller fragment from psaD/SK+ (≈ 1300 bp) containing the microRNA was isolated and cloned into the pChlamiRNA3int vector (large fragment of about 5200 bp).

Plasmid DNA from transformants was isolated and checked for the presence of the microRNA-specific *Afe*I site confirming the presence of the microRNAs (Figure 3.5). The original vector that does not contain a microRNA insert is not cut by *Afe*I and runs as supercoiled plasmid on the agarose gel (control).

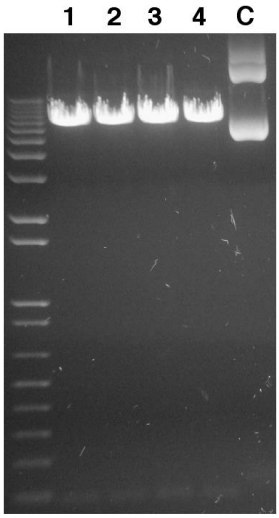


**Figure 3.5: *Afe*I digestion of plasmid DNA isolated from *E. coli* cells that have been transformed with the final transformation vector**. Lane 1, 2, 3, and 4: DNA from cells transformed with microRNA1, microRNA3, microRNA4, and microRNA6, respectively. C: control = transformation vector (no insert).

To confirm (again) the orientation of the inserted microRNAs, plasmids were digested with *Spe*I and *Xba*I which should release a fragment of ≈150 bp if the microRNAs are in the right orientation (Figure 3.6).

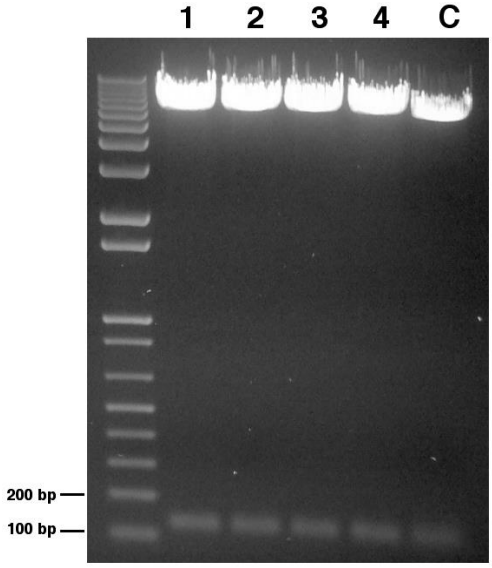


**Figure 3.6: Final constructs digested with *Xba*I/*Spe*I.** Lanes 1, 2, 3, and 4: DNA from cells transformed with microRNA1, microRNA3, microRNA4, and microRNA6, respectively. C: control = transformation vector pChlamiRNA3int without insert.

Maxipreps of the plasmids harboring the four microRNAs were made and linearized by digestion with *Kpn*I. *Chlamydomonas* cells were transformed by the glass beads method (Materials and Methods) and plated on agar containing 60 µg/ml paromomycin. Individual colonies could be seen on the plates about 4 weeks after transformation.

## 3.2 Screening of transformants by PCR

Individual colonies of *Chlamydomonas* were picked and grown on liquid HS medium to a cell density of about 1-5 million cells per ml. Total DNA was isolated and screened by PCR for the presence of the paromomycin gene and the microRNAs. PCR primers used, binding sites, and expected sizes of PCR products are indicated in Figure 3.7.

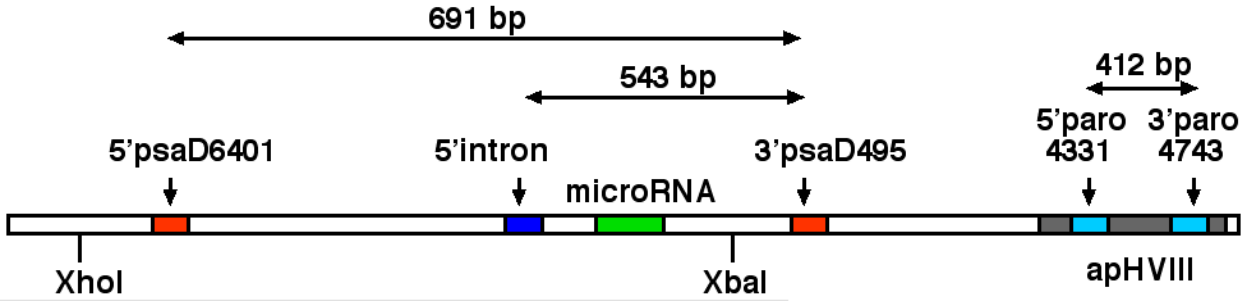


**Figure 3.7: Map of the linearized final transformation vector in which the primer binding sites are marked.** Sizes of the expected PCR products are as follows: 5'psaD6401/3'psaD495, 691 bp. 5'intron/3'psaD495, 543 bp. 5'paro4331/3'paro4743, 412 bp. apHVIII, paromomycin resistance gene. The map is not drawn to scale.

The presence of the paromomycin resistance gene in the nuclear genome of *Chlamydomonas* was checked with primers 5'paro4331 and 3'paro4743 yielding a PCR product of 412 bp (Figure 3.7). All transformants checked contained the paromomycin resistance gene (Figure 3.8) which was not surprising because they have been selected by growing on paromomycin plates. For one of the cell lines, the presence of the paromomycin gene was also confirmed by Southern analysis (see 3.3 and Figure 3.11).

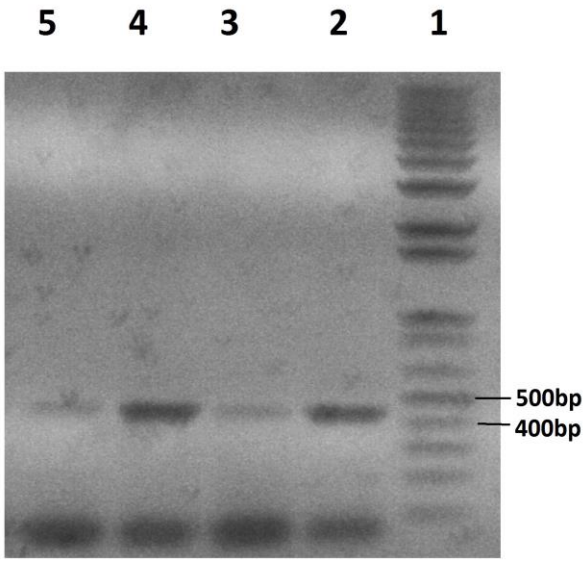


**Figure 3.8: PCR products from primers 5`paro4331 and 3`paro 4743.** The primers amplify a 412bp fragment.
Lanes 2, 3, 4, and 5: template DNA from cells transformed with microRNA1, microRNA3, microRNA4, and microRNA6, respectively.
Lane 1: 1 kb plus DNA ladder.

Two PCR reactions were done to verify the presence of the microRNA sequence in the nuclear genome of the transformants. A PCR with primer pair 5' intron/3'psaD495, with an expected fragment size of 543 bp (Figure 3.7), and a second PCR with primer pair 5'psaD6401/3'psaD495, with expected fragment sizes of 691 bp and 778 bp, the latter being a PCR product from the endogenous *psaD* gene in the genome. The results of these PCRs are shown in Figures 3.9 and 3.10.

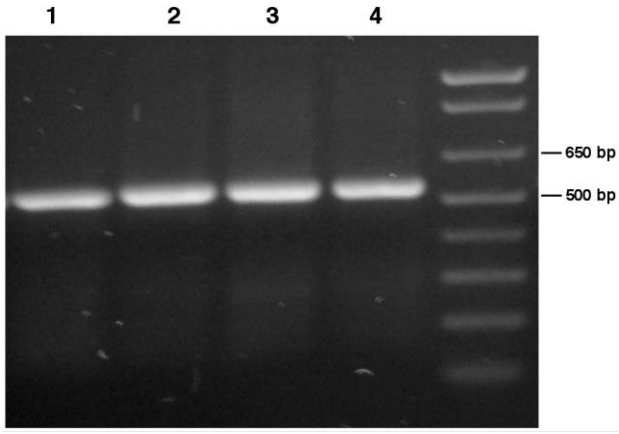


**Figure 3.9: PCR products from primers 5` intron and 3`psaD495.** Expected fragment size is 543 bp.
Lanes 1, 2, 3, and 4: template DNA from cells transformed with microRNA1, microRNA3, microRNA4, and microRNA6, respectively.

Besides the expected products of 691bp and 778 bp, PCR reactions with primer pair 5'psaD6401/3'psaD495 also gave a product of around 600 bp (lowest band in Figure 3.10). This is assumed to be an unspecific amplification product from unspecific binding of the primers to the genomic DNA.

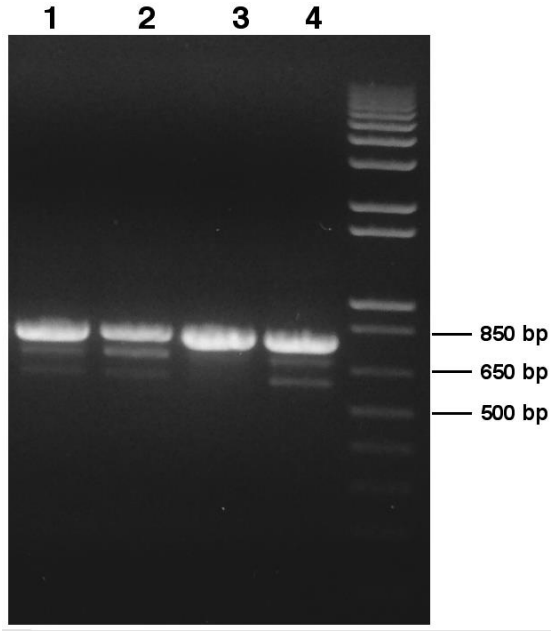


**Figure 3.10: PCR products from primers 5` psaD6401 and 3`psaD495.** Expected fragment sizes are 691 bp and 778 bp (from the endogenous *psaD* gene. Lanes 1, 2, 3, and 4: template DNA from cells transformed with microRNA1, microRNA3, microRNA4, and microRNA6, respectively.

Of the four transformant cell lines, the microRNA4 transformant did not give the expected fragment of 691 bp in PCRs with primer pair 5'psaD6401/3'psaD495. Because of this result and because of time constraints the following analyses were only done with the microRNA1 transformant.

## 3.3 Detecting the paromomycin resistance gene by Southern analysis

In addition to PCR, the presence of the paromomycin resistance gene in *Chlamydomonas* genomic DNA was confirmed by Southern analysis. Genomic DNA from transformant microRNA1 was digested with *Bam*HI and *Xho*I and separated on an agarose gel (Figure 3.11 a).

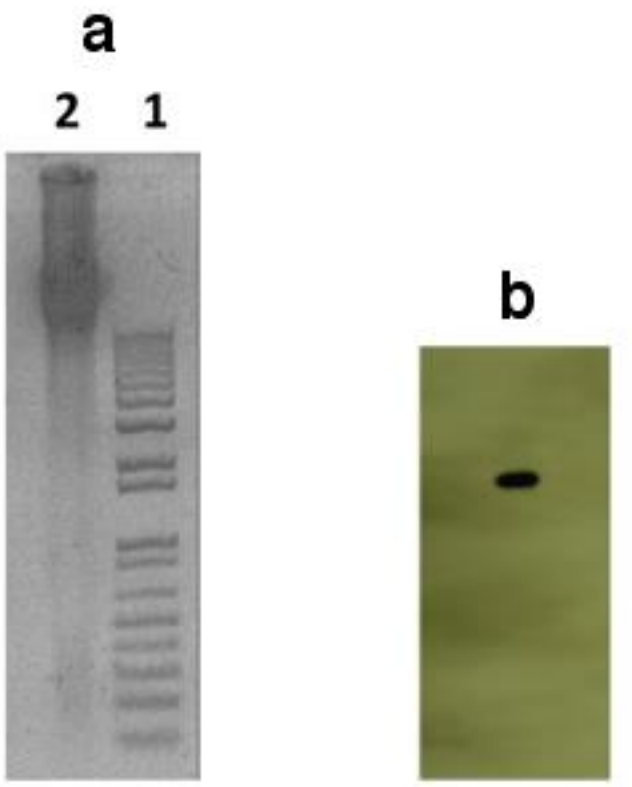


**Figure 3.11: Southern analysis of genomic DNA isolated from a cell line transformed with microRNA1.**
**a.** DNA digested with *BamH*I and *Xho*I and separated on an agarose gel (lane 2).
**b.** autoradiogram of the DNA shown in a. after blotting to a nylon membrane. The probe was the random primer-labeled coding sequence of the paromomycin resistance gene (*apHVIII*). The x-ray film was exposed to the membrane overnight.

After transfer of the DNA to a nylon membrane it was hybridized to a probe specific for the *apHVIII* coding sequence. A single band appeared on the autoradiogram indicating the presence of a single copy of the paromomycin resistance gene in the *Chlamydomonas* genome.

## 3.4 Effect of microRNA1 on expression of the *rppH* gene

In order to assess whether microRNA1 has an effect on expression of the *rppH* gene in transformant cells levels of *rppH* mRNA were determined by Northern analysis. Transformant cells harboring the microRNA1 gene and CW-15 cells (control without microRNA) were grown in 12 hour light/12 hour dark cycles and total RNA isolated at 11 hours in the dark. The RNA was fractionated on a formaldehyde gel (Figure 3.12 a) and transferred to a nylon membrane. The membrane was hybridized to a random primer-labeled probe specific for the coding sequence of the putative *Chlamydomonas rppH* gene (Figure 3.12 b).

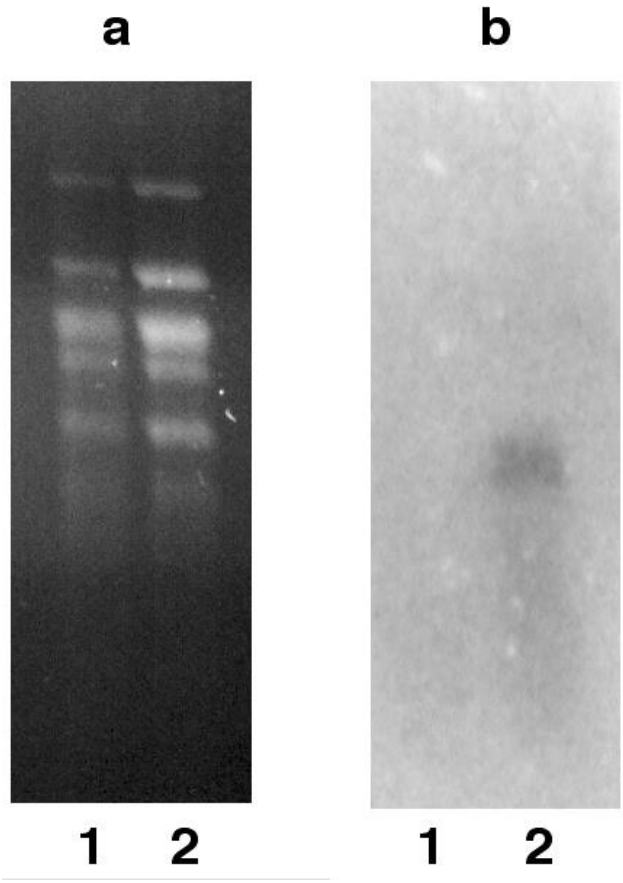


**Figure 3.12: Agarose gel with total RNA from the microRNA1 transformant (lane 1) and from CW-15 cells (lane 2).**
**a.** Photo of the agarose gel.
**b.** Autoradiogram showing a fuzzy band (possible two bands) only in the lane containing the CW-15 RNA.
Note the different amounts of RNA in the two lanes in **a**.

*RppH* mRNA was only detected in CW-15 (control) cells in the analysis indicating that microRNA1 inhibited expression of the *rppH* gene in the transformant. However, much less transformant RNA was loaded onto the gel (Figure 3.12 a) making it impossible to interpret the result of the Northern analysis. Unfortunately, there was no time left to repeat the analysis.

# 4. Discussion

In this project four artificial microRNAs, targeting sequences in the untranslated regions of transcripts of a putative *Chlamydomonas rppH* gene, were inserted into the nuclear genome of CW-15, a cell wall-less *Chlamydomonas* mutant. The presence of the artificial microRNAs in nuclear transformants was checked by PCR. Due to time constraints, only one of the transformant cell lines was analyzed for an effect of the artificial microRNA on expression of the *rppH* gene.

## 4.1 MicroRNAs

Four different microRNAs were used in this project. All four microRNAs bind to the 5` and 3` UTR regions of the mRNA. None of them is specific for the exon regions of the *rppH* gene. The logic behind using these four miRNA is as follows: there are at least eight other genes with a Nudix motif in the *Chlamydomonas* genome. If selected microRNAs were specific for exon sequences, they could silence other genes containing the Nudix motif as well increasing the chance to generate non-viable transformants.

## 4.2 Nuclear transformation

The glass beads method of nuclear transformation method was used to transform *Chlamydomonas* with microRNA 1, 3, 4, and 6. MiRNAs are inserted in random locations into the *Chlamydomonas* genome. Upon successful transformation *Chlamydomonas* cells can grow on paromomycin plates. *Chlamydomonas* transformants from plates were selected for DNA isolation and their DNA was screened by PCR for the presence of the paromomycin resistance gene and the presence of the microRNA sequences. However, expression of the artificial microRNAs in transformants was not shown directly, e.g. by using a probe specific for the microRNAs or by RT-PCR. Therefore it is not sure that the microRNA genes are actually transcribed and processed correctly such that they are functionally active. Such an analysis would require more resources than have been available in the project.

## 4.3 Southern analysis

Southern blotting was used to check if the vector is inserted into the *Chlamydomonas* genome. DNA from *Chlamydomonas* with microRNA1 was isolated and digested with *Bam*HI and *Xho*I. Digested DNA then was fractionated on an agarose gel. The gel was inspected under UV light and the DNA was transferred to a membrane. A probe specific to the sequence of the aminoglycoside 3`phosphotransferase gene (*apHVIII)* responsible for paromomycin resistance was hybridized to the membrane. After exposure to an x-ray film the presence of a strong band on the film proved that the paromomycin resistance gene was inserted. Most likely the other sequences in the transformation vector, including the microRNA genes, have been inserted into the genome as well.

## 4.4 Northern analysis

Total RNA was isolated from one of the transformants and from the wild type. RNA from the microRNA1 transformant was fractionated on a gel and transferred to a membrane. Later an *rppH* probe was added to the membrane and the film was developed.

*RppH* mRNA could only be detected in CW-15 (control) cells that did not harbor an artificial microRNA gene. This seems to indicate that the microRNA in the transformant does indeed silence expression of the *rppH* gene. However, as less mRNA from the transformant than for the wild-type was used in the analysis, no such conclusion can be drawn. The analysis needs to be repeated with equal amounts of total RNA.

In addition to Northern analysis, qRT-PCR could be used to estimate the levels of *rppH* transcripts in transformants and wild-type cells but there was no time to do this.

Another possible approach to assess whether expression of the *rppH* gene is silenced by the artificial microRNAs would be to analyze the level of RppH protein by immunoblots. Unfortunately, no antibodies against the *Chlamydomonas* RppH protein are available and production of antibodies would take a few months.

# Conclusion

The goal of the project was to identify the function of a putative RNA pyrophosphohydrolase gene in *Chlamydomonas reinhardtii* by silencing the expression of the gene and analyzing the effect of silencing on mRNA degradation. In this work artificial microRNA genes, targeting transcripts of the putative *rppH* gene, were inserted into the nuclear genome of *Chlamydomonas.* Preliminary analysis by northern blotting suggests an effect of one of the microRNAs on *rppH* transcript levels indicating functioning of the artificial microRNAs in silencing expression of the target gene. However, the analysis needs to be repeated and additional analyses are needed to come to a clear conclusion. If silencing is confirmed by additional analyses, the effect of *rppH* silencing on mRNA levels has to be determined in order to verify that the putative *rppH* gene is indeed an RNA pyrophosphohydrolase.

# Future perspectives

Only one cell line transformed with an artificial microRNA has been analyzed in the project. As the results of this analysis are not conclusive, cell lines harboring the other three artificial microRNAs should be analyzed too.

Transformants in which expression of the putative *rppH* gene is silenced should be analyzed for an effect of silencing on mRNA degradation. RppH is thought to initiate mRNA degradation in bacteria by removing pyrophosphate from the 5' terminus of mRNAs. Mutant bacterial cell lines that do not produce a functional RppH protein have been found to have significantly higher levels of mRNAs. If the putative RppH in *Chlamydomonas* functions in the same way, levels of mRNAs should also be higher in the alga. As *Chlamydomonas* RppH is supposed to be located in the chloroplast, only chloroplast mRNA levels should be affected (nuclear mRNAs do not have triphosphates at their 5' terminus but a 5' cap).

Isolated RNA can be checked with probes specific to different chloroplast mRNAs. *RbcL* and *psaB* gene sequences are candidates for this analysis because of high abundance of their transcripts in the *Chlamydomonas* chloroplast making it possible to use traditional quantitative northern analysis.

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# Appendix 1

Details about pBlueScript II SK (+) cloning vector for cloning of the microRNA, map and method the digestion of the SK+ with SpeI.
pBlueScript II SK(+)

Vector Type: Bacterial

Viral/Non-viral: Nonviral

Promoter: lac

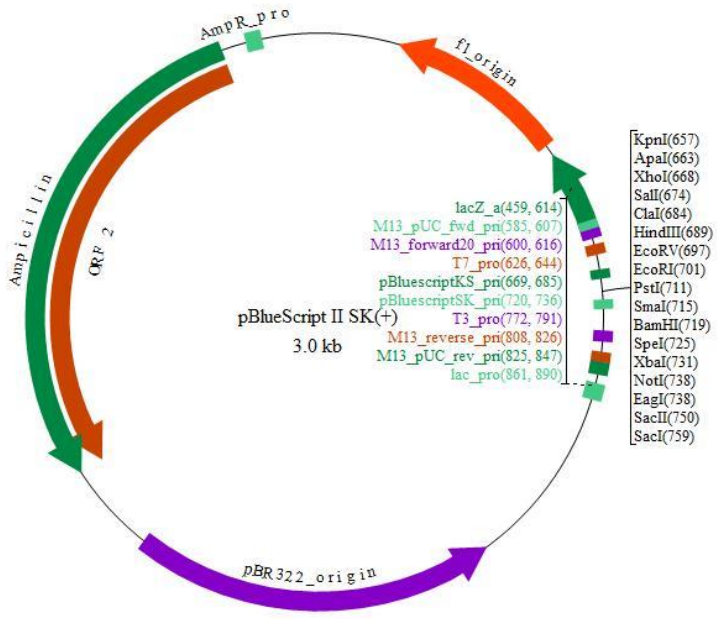
Backbone size: 3000

Sequencing Primer: T7 Fwd

Bacteria Resistance: Ampicillin

GenBank Accession Number:X52328

Comments:MCS oriented as SacI-KpnI; f1 ori can be in either orientation; contains lacZ reporter (Figure 1).




**SK+ vector. SK+ vector used for cloning in the experiment.** Ampicillin resistance gene and promoter (pBR322- origin)

# Appendix 2

Details about pChlamiRNA3int cloning vector for cloning of the microRNA, map and method the digestion of the pChlamiRNA3int with XbaI and XhoI.

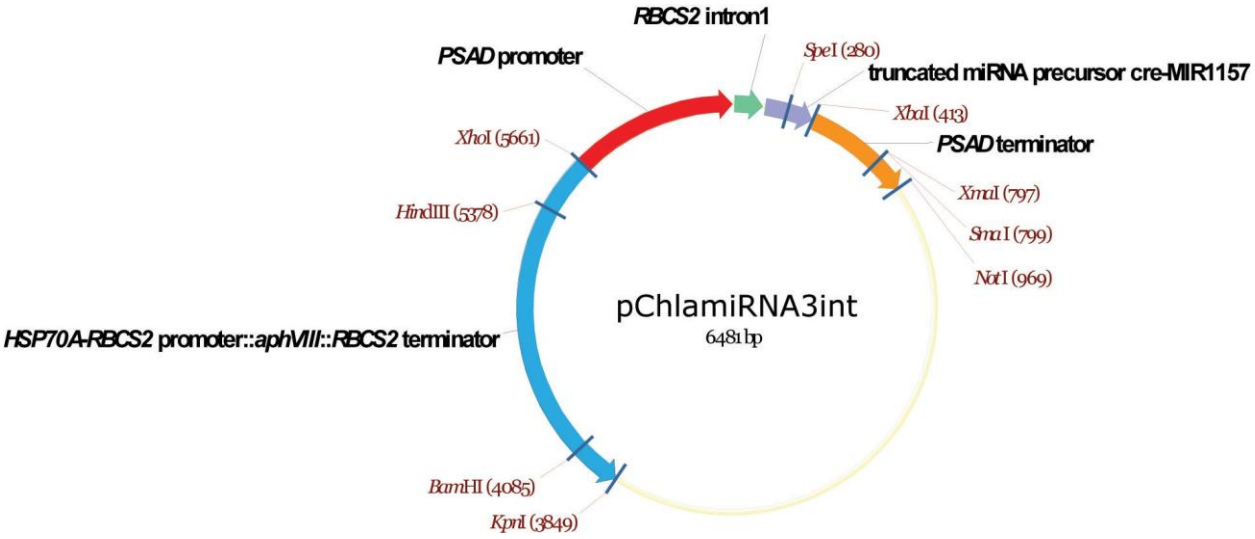



**Figure 2: pChlamiRNA3int vector.** pChlamiRNA3int plasmids used for transformation of *Chlamydomonas*. Plasmids contain *PsaD* promoter. *PsaD* is a strong promoter that is taken from plant cells.

# Appendix 3

## Sequence of the *rppH* gene with 3`and 5`UTR sequence , exon and intron.

ATATCGTAGGCTTGGGGGCCGAGTCAGGGACGGGCTCGGGGAAAACACGGCGTGTCTGCGAGATTCCGCGTGGCGCA
TTATCCCTTGCTTGCTGGGTGCCACTACAGAAACAGTCACCAGAAAACCTATCCCTGCCATCGCAAGCTTATAACCG
CGTTTGTTGGTTCTTTCTTGACAACTAGGGTCATCATCCCCTCCTGACCTGTTTGCGCTGCGCGGCCCGTCGCGAGG
CTCTCACGTCAACATATACTTGCTTTCATGCTGGCTACAAGGTGTGCGATTTTCAGGCCGTGACTCTTTCCCGCCCT
CGGTCTCGCGGAACTGCTTCATGCTCTGCTTTCTGCACAGTGACCATGTAATTTATTAAGAACGTGACACATAGTAT
CCAGTGCAAGTTGAGTGCTGCAGCTCGCGCGGGCTCTTGAGCGACTTCAGTTTGCCCTCCGAAAACCCCCGCACTGT
CGCAGGCCGCCCGTCGGTGCGCCCTCGCGGGGAGCCCTGTGCCGCGCGCCTCGCGCTCTGTCCCGCCGCTCGCTCTT
GGCCGTGCCGCCTGCTGCGGCGCCAGCCTCGGGCCCCGGACCGGCATCTTCGGGACCGCCCGGCCCGTCGCCATCCT
CGAGCGACTACATAAACCCAGCGTACTTCTACGCCAACCCCTTCGCGCCAGCTCAGGGCGCAACTTCCTCCAGCTCA
TCGCCCGGATCCAGCGGCCCCAGCTCCCACGCCACTGTCCTCCGCACCGTTGACTCGGTCGTTGCGGCCGCCAGCGC
AGCACGCGCCGCAGCCACCGCCCGCGCGGCCGTAGCGGCACGCTCTGCTTCCCCTTCCCCCTCCTCCTCGAGCGCTT
CCAGCTCTCCCGGCAGCCAGACGCACGCCCGTGCGCCCGCGGTATCCACAGCTGCTGCTGCCCCGTCTTCTTCCCCC
GCGGCCCCCGCCGCGCAGGCCGCCTCCACCGCTCCGGTGCAGCTGTCGCCTGCAGCCGCTCCTGCGCTAGCCACCAA
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TCAGCCACTACCGGGCTCAGGCCCAACTGCTTCCGGGCAACGCTTGTGCAGGCCCCTCCTGCGCGCGGCCCTGTCTT
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AGTGCCTGCGGGGGCAATGATAGCTCAGGGAAAGCAATGACAGCACACTGGGGAGCAATGATGCAATGAGTCAAGTA
GCCAAACGGGTGTTGGGGTGGGGGGACAGGAGAGGGAAAAGGGCGGGCGGGCAACACAGAAGAGTAAGGGTGCCGAG
CATGTGTGTATGGACTTGCATGTATGCCTGCGTTGGGATCACTGACTATTGATGGGCCGGTGGTGGGTTGCCAGTCC
AATTGTAGCGGGGACGGCGCCAGCCATCGGCTTTCTGCGACACATCAGAAGGATATCGTACTTGCAGTACCGCTGCG
TCTTTGGACGCAGCTATTCCTAAGCACGGCATGCAACACATGACAACAATGGAAACCAACACAGGCGGCGCTGGTTC
GCGAGCTGTATGAGGAGCTGGGCATCTCGGTGGACCCGGCGGACCTGGCGCCGCTCACATTTGCCTCCCACACCTAC
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GACAGACAGGCATGAGATCGGACGAGCTAGACGTGTGGGTCAGCAGAGATTGGTCGAGGTTCTCCCGATGTAATACG
CAGTCGGA

The green colour is 5`UTR, blue is 3` UTR, black is exon and red is intron in *rppH*. The brown color is the site of the microRNA attachment.

# Appendix 4

## PCR

Tabel 1: Primers used to amplify the paromomycin resistance gene and annealing temperature.

| Primers | Annealing temperature °C |
|---|---|
| 5`paro 4331 | 69.6 |
| 3`paro 4743 | 59.8 |

Tabel 2: Primers used to amplify the psaD vector and annealing temperature.

| Primer | Annealing temperature °C |
|---|---|
| 3`psaD 495 | 65.8 |
| 5`psaD 6401 | 63.5 |

Tabel 3: Primers used to amplify the psaD region and intron part of the vector and annealing temperature.

| Primer | Annealing temperature °C |
|---|---|
| 5`intron | 63.7 |
| 3`psaD 495 | 65.8 |

Tabel 4: Primers used in PCR and sequences.

| Primer | Sequence |
|---|---|
| 5`paro 4331 | ACGGCCGACCCGCCCCACGT |
| 3`paro 4743 | GATTCCCGTACCTCGTGTTGT |
| 3`psaD495 | GCGAAAGCCTCCGAGCTCCGAT |
| 5`psaD6401 | CGCCGAGCAAGCCAGGGTTA |
| 5`intron | GCAACGCCCGCATTGTGTCGA |

PCR condition

1. 94°C 1 minute
2. 94 °C 30 second
3. Annealing temperature 30 sec
4. 72°C 45 second

Go to step 2 45 times

| | | |
|---|---|---|
| 5. | 72°C | 10 minutes |
| 6. | 4 | ∞ |

Appendix

**Sequence of pChlamiRNA3int.**

tatgaacaagtgagtcgacgagcaagcccggcggatcaggcagcgtgcttgcagatttgacttgcaacgcccgcattgtg
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Appendix

tgatcagcacgaaacggggagctaagctaccgcttcagcacttgagagcagtatcttccatccaccgccgttcgtcagggggcaaggctc
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agggttgcaagtgctcaaataccccatcaaacatcatcctggtttggctgcgctccttctggcgcgcccggcatgcaagcttgatgggat
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ttgtgcattctaggaccccactgctactcacaacaagccca

Tabel 5: Different primers attached itself to the pChlamiRNA3int.

| Primer | Color |
|---|---|
| 5`paro 4331 | Blue |
| 3`paro 4743 | Blue |
| 3`psaD 495 | Orange |
| 5`psaD6401 | Orange |

| | |
|---|---|
| 5`intron | Green |



# Appendix 5

**MicroRNA**

Wmd3.weigelworld.org (http://wmd3.weigelworld.org) was used to suggest MicroRNA. We choose 4 different microRNA for the experiment.

Tabel 6: Sequences for microRNA1, 5` and 3`

| Micro 1 | Sequence |
|---|---|
| 5`microRNA1 | TAAATGAAGCATAATGGGCAA |
| 3`microRNA1 | TTGCCCATTATGCTTTGTTTA |

Tabel 7: Sequences for microRNA 3, 5` and 3`

| Micro3 | Sequence |
|---|---|
| 5`microRNA3 | TACATACGCGGTTATAAGCTT |
| 3`microRNA3 | AAGCTTATAACCGCGTTTGTA |

Tabel 8: Sequences for microRNA 4, 5` and 3`

| Micro 4 | Sequence |
|---|---|
| 5`microRNA4 | TATGATGACCCTAGTTGTCAA |
| 3`microRNA4 | AAGCTTATAACCGCGTTTGTA |

Tabel 9: Sequences for microRNA 6, 5` and 3`

| Micro 6 | Sequence |
|---|---|
| 5`microRNA6 | TCAATCTCCAAACTAGCGCAT |
| 3`microRNA6 | ATGCCGCTAGTTTGGAGATTGA |

To transform the *Chlamydomonas* flanking sequences were added to the MicroRNA (suggested by the site)

Appendix

Tabel 10: MicroRNA 1, 5` and 3` with flanking sequence

| microRNA 1with flanking sequence | Sequence |
|---|---|
| 5`micro1 | ctatTTGCCCATTATGCTTTGTTTAtctcgctgatcggcaccatggggg tggtggtgatcagcgctaTAAATGAAGCATAATGGGCAAg |
| 3`micro1 | ctatTTGCCCATTATGCTTTGTTTAtagcgctgatcaccaccaccccca tggtgccgatcagcggagaTAAATGAAGCATAATGGGCAAg |

Tabel 11: MicroRNA 3, 5` and 3` with flanking sequence

| microRNA 3with flanking sequence | Sequence |
|---|---|
| 5`micro3 | ctagcAAGCTTATAACCGCGTTTGTAtagcgctgatcaccaccaccc catggtgccgatcagcgagaTACATACGCGGTTATAAGCTTa |
| 3`micro3 | ctagcAAGCTTATAACCGCGTTTGTAtagcgctgatcaccaccaccc ccatggtgccgatcagcgagaTACATACGCGGTTATAAGCTTa |

Tabel 12: MicroRNA 4 , 5` and 3` with flanking sequence

| microRNA 4 with flanking sequence | Sequence |
|---|---|
| 5`micro4 | ctagtTTGACAACTAGGGTCAACATAtctcgctgatcggcaccatgg gggtggtggtgatcagcagcgtaTATGATGACCCTAGTTGTCAAg |
| 3`micro4 | ctagcTTGACAACTAGGGTCATCATAtagcgctgatcaccaccaccc ccatggtgccgatcagcgagaTATGTTGACCCTAGTTGTCAAa |

Tabel 13: MicroRNA6, 5` and 3` with flanking sequence

| microRNA 6 with flanking sequence | Sequence |
|---|---|
| 5`micro6 | ctagtATGCCGCTAGTTTGGAGATTGAtctcgctgatcggcaccatg ggggtggtggtgatcagcgtaTCAAACTCCAAACTAGCGCATg |

| | |
|---|---|
| 3`micro6 | ctagcATGCGCTAGTTTGGAGTTTGAtagcgctgatcaccaccaccc<br>ccatggtgccgatcagatcgcgagaTCAATCTCCAAACTAGCGCATa |



# Appendix 6

## DNA digestion

Concentration of the vector is 207ng/µl and 5000ng or 5 µg is needed.
5000/207=24µl.

Table 14:5µg of vector SK+ digested with SpeI to make the plasmids linearized. Mixture stays at 37°C for 1 hour

| Material | Amount µl |
|---|---|
| SK+ vector | 25 |
| Buffer 2 10X | 5 |
| BSA | 5 |
| SpeI | 1 |
| $H_2O$ | 14 |
| Total | 50 |

Ligation : To make the ligation mix appropriate amount of the vector 4200 bp (SK+) insert 90bp (microRNA) must be mixed to get the maximum.

Table 15: ligation mix must be made compeer to size and contraction of the vector and insert.

| Fragment | Size bp | Concentration ng/µl. |
|---|---|---|
| SK+ | 4200 | 66 |
| RNA | 90 | 20 |

=Size of the vector /size of the insert
=4200bp/90bp=
46.6

200ng/46.6

=4.34 ng of insert.

To make ligation mix add 200ng vector to 4.4ng insert and add water to final concentration of 6.5µl.

Appendix

Table 16: Appropriate amount of material for digestion of the SK+ with XbaI , SpeI

| Material | Amount (µl) |
|---|---|
| DNA (SK+) | 3 |
| Buffer 2 10X | 1 |
| Enzyme(XbaI , SpeI) | 0.25 , 0,25 |
| RNAase | 1 |
| BSA | 1 |
| Water | 3,5 |
| Total | 10 |

Table 17: Appropriate amount of material for digestion of the SK+ with AfeI

| Material | Amount ( µl) |
|---|---|
| Buffer 4 10X | 1 |
| RNAase | 1 |
| DNA | 5 |
| Water | 2,7 |
| Enzyme AfeI | 0.3 |
| Total | 10 |

Table 18: Digestion of the pChlamiRNA3int with XbaI and XhoI.

| Material | Amount ( µl) |
|---|---|
| Vector | 15 (12000ng ) |
| Buffer 2 10X | 5 |
| BSA | 5 |
| XbaI | 1 |
| XhoI | 1 |
| Water | 23 |
| Total | 50 |

# Appendix 7

To Remove the DNA from the gel GFX PCR DNA gel band purification kit (GE healthcare) were used.

LB medium/L
10g tryptone
5 g yeast extract
10g NaCl

LB amp/L plates
10g tryptone
5 g yeast extract
10g NaCl
15 g agar
100μg Amp

TAE (50x)/L
242g Tris base
57.1ml glacial acetic acid
100ml 0,5 M EDTA (pH 8.0)

Loading buffer
0.25% Bromphenol blue
0.25% xylene cyanol FF
30% glycerol

HS media (high salt)
For 1 liter:
20 ml salt stock
20ml phosphate stock
1ml trace element (Hunter)

Salt stock(50x):
For 500ml :
12.5g $NH_4Cl$
0.50g $MgSO_4$. 7 H2O
0.25 g $CaCl_2$. 2 H2O

## Phosphate stock (50X)

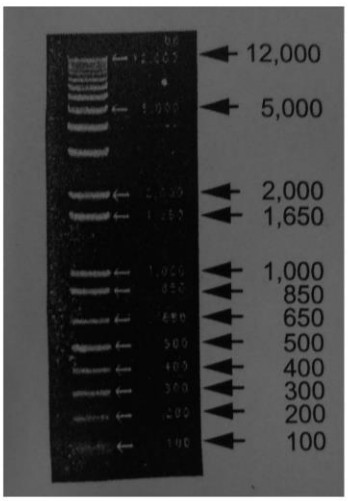
For 500ml:
47g $K_2HPO_4$. 3H2O
18g $KH_2 PO_4$

Appendix

Materials for min prep:

1,5 ml eppendorf tubes, sterile
Pipettes and tips
box with ice
tube rack for microfuge tube
TEG buffer ( 25 mM Tris-HCl, pH 8,0)
10mM Na2 -EDTA, 50 mM glucose, ice cold(1ml)
NaOH [2n] (1m)
SDS (sodium dodecyl sulfate) [10%] (1m)
Potassium acetate [5 M, pH4.8], ice -cold 5M acetate and 3 M potassium(1ml)
Phenol/chloroform /isoamyl alcohol [ratio 25:24:1] (2ml)
Chloroform /isoamyl alcohol [ratio 24:1] (2ml)
Sterile distilled water (1ml)
Absolute ethanol [ice- cold] (500ml)
70% ethanol [ice- cold] (500ml).

1




1kb

**Figure 3: 1kb ladder used in the lab as DNA size marker.**